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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. PP 6646 for a patent by SOUTHERN CROSS UNIVERSITY and RURAL INDUSTRIES RESEARCH & DEVELOPMENT CORPORATION as filed on 22 October 1998.

WITNESS my hand this
Twenty-fourth day of September 2003

J. Billingsley

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TEAM LEADER EXAMINATION
SUPPORT AND SALES

P/00/009
Regulation 3.2

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: "PLANT GENOME TRANSFER"

The invention is described in the following statement:

TITLE

"PLANT GENOME TRANSFER"

FIELD OF THE INVENTION

THIS INVENTION relates to a method of genome transfer in plants. More particularly, the method relates to transfer of a plurality of genes by microprojectile bombardment. This invention also relates to genetically-modified plants produced by the method, and in particular genetically-modified *Oryza sativa* (rice).

BACKGROUND OF THE INVENTION

The transfer of desirable phenotypic traits between plants has traditionally been performed by selective breeding. Such breeding practices have been central to the development of efficient agricultural societies. More recently, recombinant DNA technology has revolutionized breeding practices, although the overall aims of conventional breeding and recombinant DNA technology are virtually identical. In fact, in hindsight it is now evident that traditional breeding practices provided a means whereby genetically-heritable phenotypic traits were introduced into plants, although in the absence of any knowledge of the genetic basis of heritability.

The underlying principle of modern recombinant DNA technology is that phenotype correlates with genotype. The resultant practical implication to genetic engineering is that transfer of a specific phenotypic trait can be achieved by transfer of a corresponding gene. As used herein, "transfer" means introduction of one or more genes into a

plant, wherein the one or more genes are not usually present in the genome of the plant. In this regard, "genes" may include distinct allelic forms of a genetic locus. Accordingly, the one or more genes typically underlie phenotypic traits not normally exhibited by the plant.

As used herein, the plant which acts as a source of a gene is the "donor" and the plant into which the gene is introduced is the "recipient". A donor and recipient may be genetically distinct by virtue of being members of different species, or different cultivars, breeds, races, strains or individuals of the same species. The plant resulting from gene transfer from said donor to said recipient is a "genetically-modified" plant.

Generally, gene transfer methods applicable to genetic engineering have sought to achieve either transient or stable gene expression. Stable expression occurs when the transferred gene is stably integrated into the genome of a recipient plant. It is stable integration of a transferred gene which allows genetic engineering to permanently and heritably modify plant genotype, and hence phenotype.

In plants, an obstacle to both transient and stable gene expression has been the lack of a generally applicable system for gene transfer. The earliest useful method, which in fact is still widely employed, is *Agrobacterium* mediated transformation. *Agrobacterium tumifaciens* is a naturally-occurring bacterial parasite of plants which harbours a tumour-inducing (T_i) plasmid useful for introduction of genes into plants. Reference is made, for example, to International Publication No. WO84/02920 in this regard. However, *A. tumifaciens* can be very

selective in terms of infectable donor plants, and has proven to be virtually useless with monocotyledons such as cereals. This limited applicability has led to alternative means of transferring genes into plants.

Such alternative systems include electroporation, PEG-mediated transformation, microinjection and protoplast fusion. Although these systems have had some success, generally, they involve transfer of genes into cell suspensions or protoplasts, from which regeneration of plants has proven problematic (reviewed in Christou, Particle Bombardment Technology for Gene Transfer pp 71-99. Eds Ning-Sun Yang & Paul Christou, UWBC Biotechnical Resource Series, which is herein incorporated by reference)

A particularly important development has been in the area of "microprojectile bombardment", alternatively known as "biolistic", "particle" or "microparticle" bombardment. Whichever term is used, this approach generally involves coating microprojectiles with DNA obtained from a donor plant, and accelerating the DNA-coated microprojectiles with a "particle gun" into suitable cells derived from a recipient plant. The microprojectiles are usually small, dense and made of biologically inert material such as gold. The bombarded cells, usually embryonic callus, are then propagated into plants, at least some of which have DNA from the donor plant introduced into their genome.

The most important impact of particle bombardment has been to enable transfer of genes into plants normally recalcitrant to other gene transfer techniques. Many of these plants are agronomically

important, and include rice (Christou *et al.*, 1991, BioTechnology **9** 957; International Publication No. WO92/20809), wheat (International Publication No. WO94/13822) and sugarcane (Franks & Birch, 1991, Aust. J. Plant. Physiol. **18** 471).

The earliest version of microprojectile bombardment may be found in European Patent No. 331855, but given its great success, considerable effort has been made to refine this technique. One such effort has been to improve particle guns, with various incarnations being developed which include helium-driven devices (Sanford *et al.*, 1991, Technique **3** 3), a microtargeting particle accelerator (Sautter *et al.*, 1991, BioTechnology **9** 1080), a pneumatic apparatus (Iida *et al.*, 1990, Theor. Appl. Genet. **80** 813) and an electric-discharge type apparatus (Christou *et al.*, 1991, BioTechnology **9** 957).

Other efforts have focussed on broadening the type of cells or tissues which are amenable to microprojectile bombardment, and from which genetically-modified plants may be propagated. Although embryogenic callus tends to be the tissue type of choice, other tissues such as meristem, zygotic embryos, root and stem sections have also been successfully employed (as reviewed in Christou, *supra*).

Overall, the aim of genetic engineering of plants by microprojectile bombardment, and generally other methods of gene transfer, has been to transfer two genes: usually a gene of interest together with a selectable marker gene. This has been dictated by two

considerations:-

(i) the fact that for the purposes of gene transfer, genes are usually inserted into specialized expression vectors which places practical limitations on the number of genes which can be transferred; and

(ii) the underlying goal of genetic engineering to precisely introduce or modify a specific phenotypic trait by transferring a single gene known to underlie such phenotypic trait.

OBJECT OF THE INVENTION

The present inventors have realized that gene transfer methods which employ microprojectile bombardment have been exclusively directed to the transfer of single genes which underlie a known phenotypic trait. This practice has effectively limited the scope of gene transfer as a useful tool in plant breeding. Clearly, it may be desirable to transfer a plurality of genes potentially underlying a number of phenotypic traits. Furthermore, as yet unidentified genes may well underlie phenotypic traits that confer advantageous characteristics upon genetically-modified plants.

It is therefore an object of the invention to provide a method of creating genetically-modified plants whereby a plurality of genes, whether known or unknown, are transferred by microprojectile bombardment.

SUMMARY OF THE INVENTION

According to a first aspect, the present invention resides in a method of genome transfer including the steps of:-

(i) immobilizing a plurality of DNA molecules on microprojectiles, said plurality of DNA molecules being representative of a genome of a donor plant;

(ii) bombarding cells or tissues derived from a recipient plant with the DNA immobilized on microprojectiles obtained in step (i); and

(iii) selectively propagating one or more genetically-modified plants from the bombarded plant cells or tissues obtained in step (ii), wherein a genome of said one or more genetically-modified plant(s) has at least a portion of said genome of said donor plant integrated therein.

It will be appreciated that the present invention provides a method whereby at least a portion of a donor plant genome can be integrated into a recipient plant genome by microprojectile bombardment to thereby create a genetically-modified plant.

Said plurality of DNA molecules suitably comprises a plurality of donor plant genes, whether known or unknown, at least some of which may underlie phenotypic traits of said donor plant. Thus the method of the invention allows transfer of a plurality of DNA molecules which are representative of the donor plant genome to potentially provide

said genetically-modified plant with a multiplicity of phenotypic traits characteristic of the donor.

As will be described in detail hereinafter, the method of the present invention allows the creation of genetically-modified plants having significant introgressions of donor genes, such that the genetically-modified plant may effectively constitute a "hybrid" between said donor plant and said recipient plant. Furthermore, the donor and recipient plants may share only limited genetic similarity and therefore be resistant to traditional breeding methods. The present invention provides a means whereby genetically-modified "hybrids" may be produced from donor and recipient plants, such hybrids being unattainable by conventional breeding or genetic engineering techniques.

Suitably, said plurality of DNA molecules may be in the form of genomic DNA or cDNA. It will be appreciated by those skilled in the art that cDNA is complementary DNA synthesized by reverse transcriptase from an RNA template. Preferably, said plurality of DNA molecules is genomic DNA. Advantageously, said plurality of DNA molecules is a high-molecular weight fraction of genomic DNA. Preferably, said plurality of DNA molecules are not inserted into an expression vector, but instead are immobilized to said microprojectiles without any intervening cloning step.

In principle, the method of the invention is applicable to genome transfer between any donor and recipient plant, although it will be understood by the skilled person that successful transfer and stable integration of DNA generally becomes more difficult the further the donor

and recipient plant are separated taxonomically. Preferably said donor plant and said recipient plant are distinct species. Advantageously, said donor plant and said recipient plant are distinct species from separate genera. According to a preferred form of the method, said donor plant is *Zizania palustris* (wild rice) and said recipient plant is *Oryza sativa* (rice).

Suitably, said microprojectiles are in the form of small, biologically-inert particles. Preferably, said microprojectiles are in the form of 1 μM gold particles.

Suitably, said cells derived from said recipient plant are cells which are amenable to microprojectile bombardment, and are capable of being selectively propagated into plants. Preferably, said cells are embryogenic callus, although a variety of other cells or tissues may be applicable, including meristem, zygotic embryos, root and stem sections. A detailed discussion of this topic is beyond the scope of this specification, and the skilled addressee is referred to Christou, 1997, *supra* for an example of a more extensive dissertation in this regard.

Preferably, said plurality of DNA molecules immobilized onto said microprojectiles includes a gene encoding a selectable marker. Said selectable marker gene may be selected from the group consisting of a hygromycin resistance gene (*hmr*); a β-glucuronidase (*GUS*) gene; a neomycin phosphotransferase II (*npt II*) gene; a phosphinothricin acetyl transferase (*bar)* gene; a dihydrofolate reductase gene (*dhfr*) gene; a 5-enolpyruvylshikimate-3-phosphate synthase (*epsp*) gene; a chloramphenicol acetyl transferase (*cat*) gene; a 3'-adenylyltransferase

(*aadA*) gene; an acetohydroxyacid synthase (*ahas*) gene; a nopaline synthase (*nos*) gene; and a luciferase (*lux*) gene.

Preferably, the selectable marker gene is *hmr*, which gene encodes hygromycin phosphotransferase and thereby confers an ability to grow in the presence of normally toxic concentrations of hygromycin. It will be appreciated that some said selectable marker genes allow selection by addition of selective agents such as hygromycin, kanamycin or G418 (*npt II*) or glyphosate (*epsp*), while others allow selection via screening of tissue samples, such as *lux* and *GUS*. a more detailed discussion of selectable marker genes and appropriate selective agents is provided in Christou, 1997, *supra*.

Preferably, the selectable marker gene is expressed from a plasmid such as pGL2.

Central to the method of the invention is the transfer of at least a portion of a donor plant genome into a recipient plant genome. As used herein, "at least a portion" refers to 0.01% or more of a donor plant genome being incorporated. Preferably, 0.1% or more of said donor plant genome is incorporated, more preferably 1.0% or more is incorporated and advantageously 10% or more. It will be appreciated that typical plant genomes may include somewhere between 20,000 and 50,000 genes. Thus, even if only 0.01% of the genome is incorporated, this may constitute between 2 and 5 donor genes.

In order to estimate how much of said genome has been incorporated, it is preferred that Amplified Fragment Length

Polymorphism (AFLP) analysis is used for this purpose (Vos *et al.*, 1995, Nucl. Acid. Res. **23** 4407, which is herein incorporated by reference). However, other methods are also contemplated. These include Rapid Amplification of Polymorphic DNA (RAPD; Williams *et al.*, 1990, Nucl. Acid. Res. **18** 6531, which is herein incorporated by reference), Restriction Fragment Length Polymorphism (RFLP; Nathans & Smith, 1975, Ann. Rev. Biochem. **46** 273, which is herein incorporated by reference) and microsatellite analysis, such as described in Roder *et al.*, 1995, Mol. Gen. Genet. **246** 327 and reviewed in Powell *et al.*, 1996, Trends Plant Sci. **7** 215, which are both herein incorporated by reference.

A specific example of an estimate by AFLP analysis is provided hereinafter. A generalized hypothetical example follows the reasoning that if a donor genome comprises *x* genetic markers unique thereto, and a genetically-modified plant selectively propagated from bombarded callus comprises 0.03 *x* said genetic markers, then "at least a portion" equates to 3%. Such an estimate assumes that on average, each said genetic marker is linked to an equal sub-portion of the donor genome, that each said genetic marker is equally capable of incorporation into the recipient genome, and that each said genetic marker is equally detectable once so incorporated. In the absence of evidence to the contrary, these assumptions are maintained for the purposes of this specification.

According to a second aspect, the present invention resides in a genetically-modified plant having at least a portion of a donor plant

genome integrated into a genome thereof.

Preferably, the genetically-modified plant is of the species *Oryza sativa*. Most preferably, said genome of said genetically-modified *Oryza sativa* plant has at least a portion of a *Zizania palustris* genome integrated therein.

It will also be appreciated that according to this aspect, fruit, seeds, material suitable for reproductive or vegetative propagation and other cells or tissues of said genetically-modified plant are contemplated.

EXPERIMENTAL

1. MATERIALS AND METHODS

1.1 *Preparation of genomic DNA*

Zizania palustris (wild rice) leaf material was obtained from commercial field crops in southern New South Wales, Australia. DNA was isolated from approximately 5 gram of young leaves essentially according to the procedure of Weining & Langridge, 1991, Theor. Appl. Genet. **82** 209 which is herein incorporated by reference.

1.2 *Transformation*

Embryonic callus cultures from the rice cultivar Jarrah were derived from mature seeds and used as targets for microprojectile bombardment by a particle inflow gun (Finer *et al.*, 1992, Plant Cell Rep. **11** 323). The generation of embryonic callus suitable for bombardment, and the bombardment procedure using the DuPont Biolistics PDS-1000/He apparatus, are described in detail in Abedenia *et al.*, 1997, Aust. J. Plant Physiol. **24** 133, which is herein incorporated by reference.

Briefly, 8 µg of high molecular weight *Z. palustris* genomic DNA and 2 µg of plasmid pGL2 (Shimamoto *et al.*, 1989, Nature **338** 274) encoding the hygromycin-resistance (*hmr*) gene (at a 1:1 molar ratio) in 10 µl was immobilized on microprojectiles in the form of 1 µM gold particles, by a method originally described by Sanford *et al.*, 1993, Meth. Enzymol. **217** 483 which is herein incorporated by reference. 5 µl of this microprojectile preparation was used per shot by the particle inflow gun. Optimal conditions for microprojectile bombardment were established using the *GUS* (β-glucuronidase) system as previously described (Abedenia *et al.*, 1997, *supra*).

To enhance embryogenesis, callus was cultured on osmotic medium (MSC together with 0.2 M sorbitol and 0.2 M mannitol; Vain *et al.*, 1993, Plant Cell Reports **12** 84) for 4 hrs prior to bombardment, and following bombardment, was transferred to MSC medium supplemented with 30-50 $mg.L^{-1}$ Hygromycin B (Sigma). Selection was applied throughout callus proliferation, regeneration and plant propagation. Once selectively propagated plants had reached 10-15 cm in height, they were transferred to soil in a glasshouse.

1.3 <u>*Analysis of genome transfer*</u>

In order to semi-quantitatively monitor the extent of genome transfer into selectively-propagated plants, Amplified Fragment Length Polymorphism (AFLP) analysis was performed (Vos *et al.*, 1995, *supra*). AFLP analysis was conducted on genomic DNA extracted from 200 mg of leaves (extract made as previously described) obtained from plants

selectively propagated from microprojectile-bombarded callus. Reactions were performed using a Perkin Elmer AFLP Plant Mapping kit according to the manufacturers instructions (PE Applied Biosystems: AFLP Plant Mapping, 1996, pp 2-31, which is herein incorporated by reference). A brief synopsis of the AFLP analysis method is provided in sections 1.3.1 to 1.3.5.

1.3.1 *Preparation of genomic DNA*

Extracted DNA was digested with the restriction endonucleases *Mse I* and *Eco RI*, and *Mse I* and *Eco RI* adaptors ligated in a single reaction for 2 hr at 37°C. Adaptor-ligated DNA was then diluted in TE and stored at -20°C.

1.3.2 *Adaptors and Primers*

All adaptors and primers were supplied by the manufacture, and only limited primer and adaptor sequence information is provided by the manufacturer. Table 1 lists all of the primer combinations provided by the manufacturer and those used in this study.

1.3.3 *Preselective Amplification*

Preselective amplification reduces the overall complexity of amplification by making target sequences the predominant species. The sequence of the adaptors, *Mse I* and *Eco RI* restriction sites and sequences adjacent to the restriction sites serve as primer binding sites for preselective amplification. Reactions involved use of AFLP Preselective Primer pairs provided by the manufacturer to amplify from the adaptor-ligated DNA prepared at 1.3.1. Thermal cycling was

performed using PE 9600 Thermal Cycler under the following conditions:-

1 cycle at 72°C for 2 min; then

20 cycles at 94°C for 1 min; 56°C for 0.5 min and 72°C for 2 min; then

1 cycle at 60°C for 30 min; then

hold at 4°C.

Preselective amplification products were diluted in TE and stored at 4°C.

1.3.4 *Selective Amplification*

Reactions were performed using Preselective amplification products, *Mse I* primer (5 µM) and *Eco RI* dye (FAM) primer (1 µM) together with manufacturer-supplied reaction mix. Thermal cycling was performed using a PE 9600 Thermal Cycler according to the cycling conditions shown in Table 2.

1.3.5 *Detection of AFLP products*

Detection of AFLP fragments containing the FAM-labeled *Eco RI* primer was achieved by capillary electrophoresis using an ABI Prism 310 GeneAnalyser. The data were analysed using dedicated GeneScan Analysis and Genotyper software.

2. ***RESULTS & DISCUSSION***

Introgression of genes from plant-to-plant is usually limited to plants of the same species or genus (Harlan & de Wet, 1971, Taxon **20** 509). Although in rice, interspecific hybrids between *Oryza* species may produce fertile offspring (Naredo *et al.*, 1997, Genet. Res. Crop Evol. **44** 17), hopes that more distant taxa may have genetic material which might

be introduced into *Oryza* and confer improvements which are both stable, and heritable have not been realized. In this regard, reported hybrids between rice and the distant relative *Portersia coarcata*, recovered by embryo rescue, were not fertile (Jenna, 1994, Curr. Sci. **67** 744).

Rice was the first cereal to successfully be transformed, in which case transformed rice plants were regenerated from transformed protoplasts (Shimamoto *et al.*, 1989, Mol. Breed. **4** 99). Subsequent protocols have been developed which enable rice transformation by microprojectile bombardment of callus derived from mature embryos of commercial rice cultivars (Christou, 1997, Plant. Mol. Biol. **35** 197; WO 92/20809; Abedenia *et al.*, 1997, *supra*). Traditional transformation by microprojectile bombardment has focussed on the transfer of a single gene of interest, usually in combination with a selectable marker gene. For example, the method disclosed in International Publication No. WO92/20809 described transformation with the Bar gene inserted into the plasmid pCMC2114 which encodes the *GUS* marker.

Although, the present inventors used microprojectile bombardment as a starting point for genome transfer into rice, their aim was not to merely transfer a single gene, but to transfer a plurality of genes which represent a substantial portion of a donor plant genome. The intended purpose was to genetically-modify plants so as to display multiple genetically-encoded characteristics of the donor. Ess ntially, this would amount to creation of a genetically-modified "hybrid", by an approach entirely distinct from the traditional process of cross-breeding

and outside the capabilities of conventional microprojectile bombardment approaches. Importantly, although the donor plant *Z. palustris* was known to display distinct phenotypic traits such as cold-tolerance, the genetic basis for this remains a mystery.

The present inventors isolated high-molecular weight genomic DNA from the wild-rice species *Z. palustris* (donor) as a representative sample of the genome of *Z. palustris* for transfer into *O. sativa* (recipient) callus. The genomic DNA was not inserted into a plasmid vector, but rather was directly immobilized (together with a plasmid encoding hygromycin phosphotransferase) onto 1 μM gold particles. Transgenic plants were regenerated from bombarded callus under conditions which selected for resistance to hygromycin. Fifteen (15) bombardments of 20 callus targets resulted in the successful regeneration and transfer to the glasshouse of more than 250 rice plants. The plants showed considerable morphological variation consistent with the introgression of genes from *Z. palustris*. An example of morphological variation between recipient (Jarrah) and genetically modified plants is shown in FIG. 1. A typical AFLP analysis using capillary electrophoresis and fluorescence detectionis shown in FIG. 2.

AFLP analysis indicated the presence of *Z. palustris* DNA in a significant proportion of plants tested. Analysis of 151 plants with AFLP primer pair #1 demonstrated the presence of 10 different AFLP bands from *Z. palustris* in 7 different individuals (see Table 4). The range of introgression was from 1.7% to 6.7%. That is, 1.7% to 6.7% of Z.

palustris-specific AFLP markers were present in the 7 individuals tested, suggesting that in a number of plants genetically-modified according to the method of the invention, a significant portion of the donor plant genome had been transferred.

Further analysis of 37 individual plants, chosen on the basis of their unusual appearance, with AFLP primer pair #4 revealed 20 *Z. palustris*-derived AFLP bands in 9 of the 37 plants. As shown in Table 3, one *O. sativa* plant had 16 of a possible 122 *Z. palustris*-specific AFLP markers identifiable following amplification with three different primer pairs. This equates to approximately 13% of the *Z. palustris* genome being transferred to this plant and strengthens the conclusion that many of the genetically-modified plants of the invention may contain significant introgressions of the *Z. palustris* genome.

However, it should be noted that control experiments indicated that a small number of new markers were being generated due to genetic change during the transformation process. Analysis of 4 control plants with primer pair #4 revealed a single novel AFLP product which had no apparent counterpart in *Z. palustris*. Fertility of the transgenic plants varied, but all plants set some seed. The level of introgression suggests that screening of moderate sized populations might allow the recovery of rice plants with preferred phenotypic traits. In principle, populations of hundreds or thousands of plants can be generated by the method of the invention and used as a modified germplasm resource. Detailed molecular analysis of each transgenic rice plant will be

necessary to establish the number of sites of insertion of *Z. palustris* DNA and the size of *Z. palustris* DNA inserts. Southern blotting was not a reliable method for analysis of introgression because of the relatively high sequence similarity between *Z. palustris* and *O. sativa* DNA interfering with hybridization.

Wild members of *Oryzae* have been shown to be important sources of genes for improvement of yield (Xiao *et al.*, 1996, Nature **384** 223). However, introgression of genes from wild relatives such as *Z. palustris* to *O. sativa* has not been possible due to the considerable phylogenetic distance between such family members. In fact, this "distance limitation" constitutes a fundamental barrier to creating hybrid plants throughout the plant world (Sharma, 1995, Euphytica **82** 43). Given the success of the present invention with regard to *Z. palustris*, other relatives of rice at a similar phylogenetic distance from *O. sativa* may be applicable to the method. Such a relative might be *Potamophila parviflora*.

Gene transfer by microprojectile bombardment has generally shown only minimal alteration of the rice genome, suggesting that the introduction of new genes can be achieved while maintaining genetic integrity (Arencibia *et al.*, 1998, Mol. Breed. **4** 99). Thus, in commercial crops such as *O. sativa* which can display low levels of genetic diversity, major genetic introgression from wild relatives should not destroy desirable existing traits, but should improve genetic diversity and enhance resistance to pests and other environmental contingencies.

Gene transfer by microprojectile bombardment has traditionally involved the transfer of one or two genes (usually a gene of interest together with a selectable marker) into cells or tissues derived from a recipient plant. This technique has therefore been used to transfer a known gene of interest for the purpose of transferring a particular trait. The present invention expands the usefulness of microprojectile bombardment as a means of gene transfer into the realm of creating hybrid plants. In principle, the donor plant genome may essentially be uncharacterized, but because of the significant introgression of donor plant genes according to the method of the invention, there is high probability that a number of genes are transferred to the genetically modified plant, which genes may underlie desirable phenotypic traits.

TABLES

TABLE 1

	-CAA	-CAC	-CAG	-CAT	-CTA	-CTC	-CTG	-CTT
-AA				#3				
-AC								
-AG								
-TA							#2	
-TC								
-TG		#4	#1					
-TT								

TABLE 2

HOLD	CYCLE	HOLD	NO. OF CYCLES
94°C for 2 min	65°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	64°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	63°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	62°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	61°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	60°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	59°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	58°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	57°C for 30 sec	72°C for 2 min	1
94°C for 1 sec	56°C for 30 sec	72°C for 2 min	23
60°C for 30 min	-	-	1
4°C hold	-	-	-

TABLE 3

AFLP PRIMER PAIR	NUMBER OF AFLP MARKERS IDENTIFIED	TOTAL *Z. palustris* SPECIFIC AFLP MARKERS	% OF *Z. palustris* AFLP MARKERS
#1	3: (171 bp; 211 bp; 220 bp)	60	5
#2	4: (82 bp; 111 bp; 148 bp; 496 bp)	33	12
#3	9: (221 bp; 307 bp; 329 bp; 339 bp; 348; 356 bp; 384 bp; 396 bp; 466 bp)	29	31
TOTAL:	16	122	13

TABLE 4

PLANT NO.	NUMBER OF AFLP MARKERS IDENTIFIED	% OF *Z. palustris* AFLP MARKERS
X1-18	3; 171 bp; 211 bp; 220 bp	5.0
X1-37	1; 212 bp	1.7
X4-8	1; 372 bp	1.7
X4-9	1; 372	1.7
X5-31	3; 171 bp; 242 bp; 248 bp	5.0
X5-39	2; 171 bp; 248 bp	3.3
X5-48	4; 171 bp; 248 bp; 416 bp; 442 bp	6.7

LEGENDS

TABLE 1

AFLP Primer Combinations. All primers were provided in AFLP Plant Mapping Kit. Column headings refer to *Mse I* primers and row headings refer to *Eco RI* primers. Numbers refer to primer pairs used as referred to in specification and Tables.

TABLE 2

Selective amplification conditions for thermal cycling.

TABLE 3

AFLP marker analysis of plant X1-18 using three different primer pairs.

TABLE 4

Analysis of seven individual genetically-modified plants using AFLP primer pair #1. The total number of AFLP bands present in *Z. palustris* were 118, of which 60 were *Z. palustris* -specific.

FIG. 1

Morphological differences in grains of *O. sativa* plants genetically-modified according to the method of the invention by bombardment with *Z. palustris* genomic DNA. Rice grains from the recipient rice cultivar Jarrah compared with genetically-modified rice designated X1-17 and X1-18.

FIG. 2

AFLP analysis of *O. sativa* plants genetically-modified according to the method of the invention by bombardment with *Z. palustris* genomic DNA. a: AFLP profile for *O. sativa* (Jarrah); b: AFLP profile for genetically-

modified plant X4-8; c: AFLP profile for genetically-modified plant X4-9; and d: AFLP profile for *Z. palustris*.

DATED this twenty-second day of October 1998.

SOUTHERN CROSS UNIVERSITY and

RURAL INDUSTRIES RESEARCH & DEVELOPMENT CORPORATION,

by their Patent Attorneys,

FISHER ADAMS KELLY.

FIGURE 1

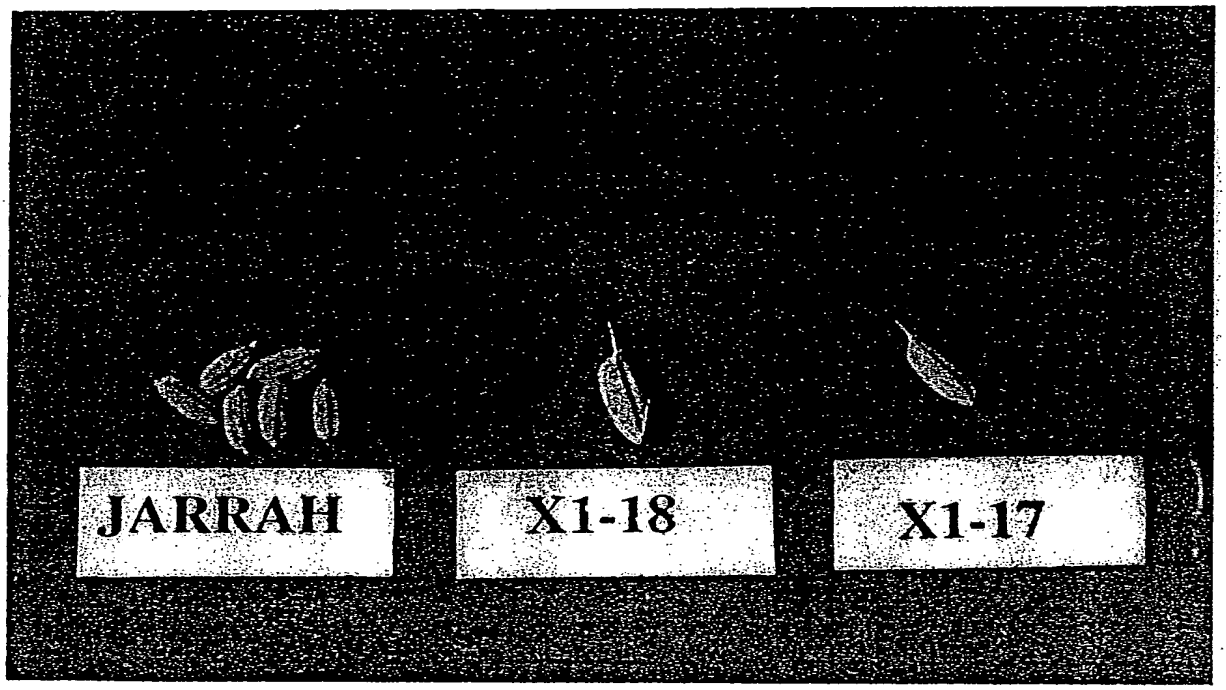
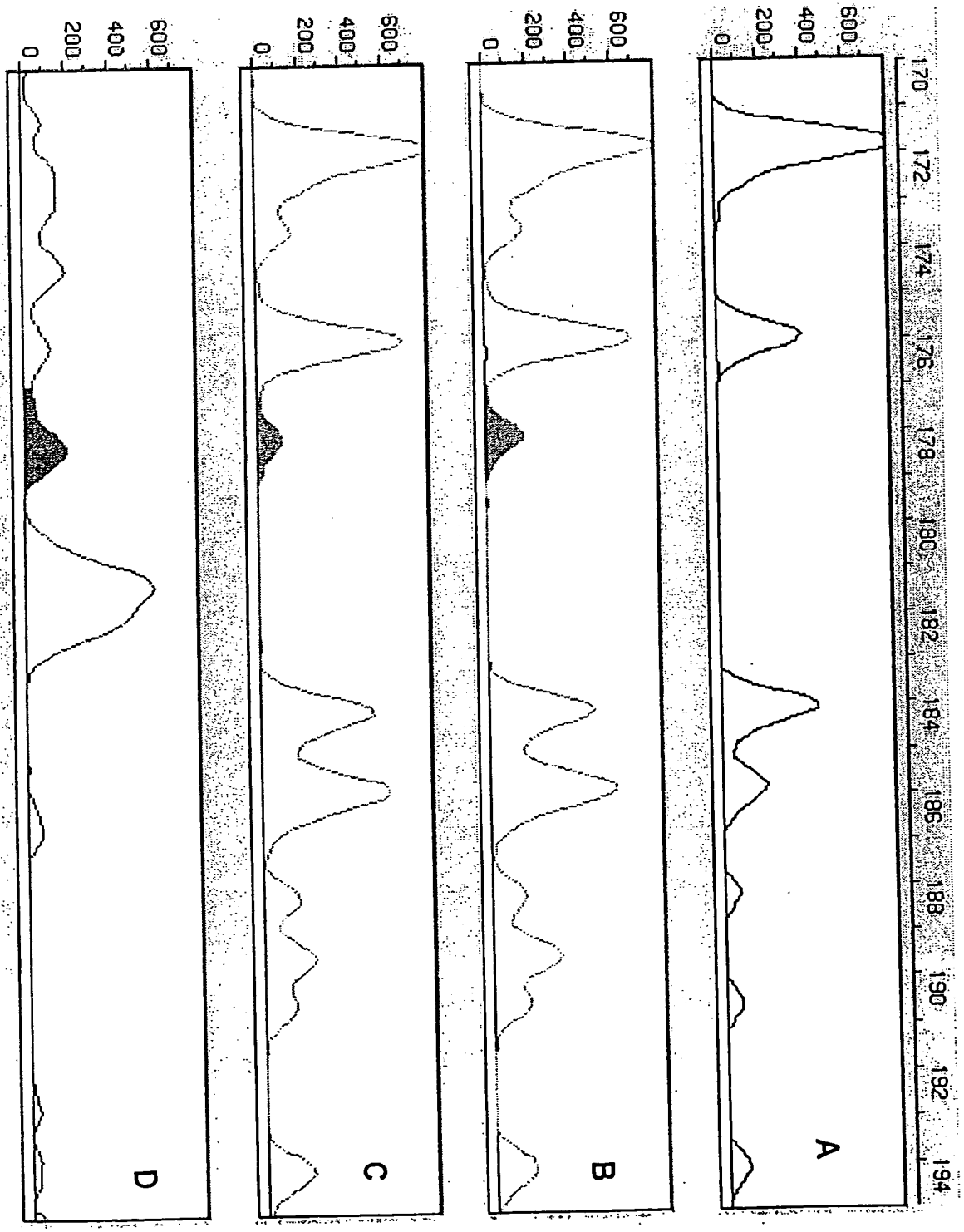


FIGURE 2


A
B
C
D
0
200
400
600
170
172
174
176
178
180
182
184
186
188
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192
194